EMSL Analytical S.O.P.

Protocol for Preparing Reagents Plates and Mixes for EPA1615 RT-qPCR

EPA-Contract

1.0 Statement of Work

1.1 The Statement of Work: the SOW used was prepared at 7/10/2014 and revised at 1/5/2015. It is provided by Dr. Shay Fout.

1.2 Purpose: The purpose of this exchange of materials is to determine the feasibility of enhancing EPA Method 1615 through the development of a kit format for the RT-qPCR section of the method (i.e., to determine whether there is any loss of sensitivity using reagents in kit format versus the standard method approach).

1.3 Reagents Plates and Reagents Mixes - Kit components to be supplied by EMSL to EPA:

1.3.1 96-well RT Plates: 3 of 96-well plates
1.3.1.1 RT plates (2 for running samples and one for a backup and repeat tests)

1.3.2 RT Master Mix-2: 10 tubes of 670 µL RT Master Mix-2
1.3.2.1 Sufficient RT Master Mix 2 for 3 plates

1.3.3 96-well Hepatitis G qPCR Plates: 3 of 96-well plates
1.3.3.1 PCR plates containing PCR Master Mix for hepatitis G

1.3.4 96-well Enterovirus and Norovirus Virus qPCR Plates: 2 PCR plates each containing PCR Master Mix (total 6 plates, two of each)
1.3.4.1 EV Plates for enterovirus qPCR assay: 2 of 96-well plates.
1.3.4.2 NV-GIB Plates for norovirus GIB qPCR assay: 2 of 96-well plates
1.3.4.3 NV-GII Plates for norovirus GII qPCR assays: 2 of 96-well plates

2.0 Interferences

2.1 The reagent plates and mixes are used for detection of RNA virus; precautions procedures, including using RNase free DH2O and reagents, must be in place.

2.2 Working bench and pipettes need to be wiped with RNaseZap (Cat no. AM9780, Life Tech) and air dried.

2.3 If the centrifuge tubes and other plastic supplies are not certified free of RNase, they must be treated with RNaseZap, then rinse with RNase free water for three times, and air dried under AirClean PCR Workstation.

2.4 qPCR detection is very sensitive, the preparation of the kits is under AirClean PCR Workstation with air on and the workstation must be exposure to UV light for 45 min before use.

2.5 No virus or environment water samples or DNA preps are allowed in the DNA/RNA Free Room.

3.0 Definitions

3.1 **RT:** reverse transcription, with reverse transcriptase, RNA molecules are converted into complementary DNA (cDNA).

3.2 **RT-qPCR:** viral RNA is the original target; the cDNA derived from viral RNA is used for quantitative PCR. The RT-qPCR has two-steps of reactions including RT and qPCR. When using RT-qPCR master mix, the RT-qPCR can be done in one step reaction, just adds viral RNA into PCR tube, both RT and qPCR take place in the same tube. However, there is no cDNA available for another qPCR confirmation. The one-step RT-qPCR has less handling steps, it can reduce RNase contamination.

3.3 **RT-qPCR used in EPA1615:** the viral RNA preps are used for RT first, then RT products are used for separate qPCR assays for detection of hepatitis G control, enterovirus, norovirus GIB and norovirus GII. Only two steps of RT-qPCR allow multiple assays for different viruses with limited amount of viral RNA preps.

4.0 Safety

4.1 All personnel performing preparation and/or analysis of samples must be familiar with the EMSL Chemical Hygiene Plan.

5.0 Equipment and Supplies

5.1 **ABI Veriti Thermal Cycler**

5.2 **StepOnePlus Fast Real-time PCR System, Life Tech**

5.3 **96-Well PCR Plates:** MicroAmp fast optical 96-well reaction plates with barcode (0.1 mL), PCR compatible, DNA/RNase free (Cat no. 4346906, Lot no. I2953Q512, Life Tech)

5.4 **PCR tubes for ABI Veriti Thermal Cycler:** MicroAmp fast reaction tubes with caps, 0.1 mL, PCR compatible, DNA/RNase free (Cat no. 4358297, Lot no. I1734Q7, Life Tech)

5.5 **Microcentrifuge Tubes:** 1.7 mL microcentrifuge tubes, RNase/DNase free (Cat no. 87003-294, Lot no. 411036-X23027, VWR). The microcentrifuge tube must be autoclaved before use.

5.6 Micropipette Tips: VWR sterile aerosol pipet tips for LTS pipettes, RNase Free

- **5.6.1** 1200 µL LTS tips (Cat no. 89201-530, Batch no. 4079-426C6-426C, VWR)
- **5.6.2** 1-200 µL LTS tips (Cat no. 89201-528, Batch no. 4064-420C4-420B, VWR)
- **5.6.3** 0.5 – 20 µL LTS tips (Cat no. 89201-526, Batch No. 3031-311C4-311C, VWR)

5.7 VWR 50 mL Centrifuge Tubes: 50 mL centrifuge tubes with screw caps, sterile (Cat no. 21008-241, Batch no. 4104-4340C-434D, VWR), not certified for RNase free. The centrifuge tubes must be autoclaved and treated with RNaseZap and rinsed with RNase free DH2O before use.

6.0 Reagents and Standards

6.1 RNase Decontamination Solution: RNaseZap, 250 mL (Cat no. AM9780, Ambion, Life Tech)

6.2 Ultra-Pure Distilled H2O: UltraPure DNase/RNase Free distilled water (Cat no. 10977-023, 10 x 500 mL, Life Tech), molecular biology grade

6.3 RT-PCR Grade Water: RT-PCR grade water (Cat no. AM9935, 10 x 1.5 mL, Ambion, Life Tech), certified free of nucleases, and free of nucleic acid contamination, no PCR inhibition, ready for use.

6.4 Armored Viral RNA Standards for QC Tests: Armored enterovirus and norovirus –GIB and norovirus GII will be provided by EPA.

6.5 Reagent Supplies from EPA: according to the Statement of Work, the following reagents in Table 1 will be provided by Dr. Shay Fout at EPA, Cincinnati, OH.

Table 1. Reagents from EPA for Making the kits

Ingredient	Volume needed (µL)	µL per unit	Tubes needed
Random primer	320	40	9
Hepatitis G Armored RNA	400	250	2
10X PCR Buffer II	1600	1500	2
25-mM $MgCl_2$	1920	1500	2
10-mM dNTPs	1280	1000	2
100-mM DTT	1600	100	17
RNase Inhibitor	200	250	1
SuperScript III RT	120	50	3
2X LightCycler 480 Probes Master Mix	10240	5000	3
ROX reference dye	409.6	1000	1
10 µM HepF	400	>3000	1
10 µM HepR	400	>3000	1
10 µM HepP	80	600	1
10 µM EntF	124.8	>3000	1
10 µM EntR	374.4	>3000	1
10 µM EntP	41.6	600	1
10 µM NorGIBF	208	>3000	1
10 µM NorGIBR	374.4	>3000	1
10 µM NorGIBP	104	600	1
10 µM NorGIIF	208	>3000	1
10 µM NorGIIR	374.4	>3000	1
10 µM NorGIIP	104	600	1

7.0 Reagents and Kits Preservation, Shipment and Storage

7.1 When the reagents arrive, they must be kept in -20°C or -80°C freezers as required.

7.2 The final Master Mix plates and Master Mix tubes must be kept in -80°C freezer in sealed plastic zip bags and covered with aluminum foil to prevent RNase contamination.

7.3 The shipping of the finished kits and reagent plates must be on dry ice and delivered via FedEx Express for overnight delivery.

8.0 Procedure for Preparation of the Kits and Plates

8.1 RT Master Mix-1 Plates:

8.1.1 One 50 mL centrifuge tube, autoclaved , add 10 mL of RNaseZap and close the cap, vortex for 5 min, then pour off RNaseZap; rinse the tube with 20 mL of DH2O, vortex, repeat three times; and leave the tube in AirClean PCR Workstation for air dry.

8.1.2 Add following RT Master Mix-1 reagents (Table 2) into the treated 50 mL centrifuge tube.

Table 2. Components for RT Master Mix-1

Ingredient	Volume per rxn (µL)	Final conc.	Volume per Mix (µL)
Random primer	0.8	10 ng/µL (c. 5.6 µM)	320.0
Hepatitis G Armored RNA	1.0		400.0
PCR grade water	14.7		5880.0
Total	16.5		6600.0

8.1.3 QC –RT Mix-1, see Section 9.0, if the QC tests pass, then continue.

8.1.4 Take 3 MicroAmp Fast Optical 96-well reaction plates, add 16.5 µL of RT Master Mix-1 into each well.

8.1.5 Cover with optical film, seal it.

8.1.6 Spin at 3,000 rpm for 3 min.

8.1.7 Put the RT plates in -80°C freezer unit shipping out to EPA lab.

8.2 RT Master Mix-2:

8.2.1 One 50 mL centrifuge tube, autoclaved , add 10 mL of RNaseZap and close the cap, vortex for 5 min, then pour off RNaseZap; rinse the tube with 20 mL of DH2O, vortex, repeat three times; and leave the tube in AirClean PCR Workstation for air dry.

8.2.2 Following Statement of Work, make the RT Master Mix-2 (Table 3) in 50 mL centrifuge tube.

Table 3. Components for RT Master Mix-2

Ingredients	Volume per Rxn (µL)	Final Conc.	Volume per Mix (µL)
10X PCR Buffer II	4.0	10 mM Tris, pH 8.3, 50 mM KCL	1600.0
25-mM $MgCl_2$	4.8	3 mM	1920.0
10-mM dNTPs	3.2	0.8 mM	1280.0
100-mM DTT	4.0	10 mM	1600.0
RNase Inhibitor	0.5	0.5 units/µL	200.0
SuperScript II RT	0.3	1.6 units/µL	120.0
Total	16.8		6720.0

8.2.3 Aliquot 670 µL into each autoclaved 1.7 mL microcentrifuge tube, total 10 tubes.

8.2.4 Put labels for the tubes and keep in -80°C freezer.

8.2.5 Run QC test for RT Master Mix-1 and RT Master Mix-2, see section 9.0.

8.3 PCR Master Mix for Hepatitis G (HGV) Plates: 3 of 96-well plates

8.3.1 One 50 mL centrifuge tube, autoclaved , add 10 mL of RNaseZap and close the cap, vortex for 5 min, then pour off RNaseZap; rinse the tube with 20 mL of DH2O, vortex, repeat three times; and leave the tube in AirClean PCR Workstation for air dry.

8.3.2 Add following HGV Master Mix-1 reagents (Table 4) into the treated 50 mL centrifuge tube.

Table 4. Components for qPCR Assay for HGV

Ingredient	Volume per Rxn (µL)	Final Conc.	Volume per Master (µL)
2X LightCycler 480 Probes Master Mix	10.0	Proprietary	4000.0
ROX reference dye	0.4	0.5 mM	160.0
PCR grade water	1.4		560.0
10 µM HepF	1.0	500 nM	400.0
10 µM HepR	1.0	500 nM	400.0
10 µM HepP	0.2	100 nM	80.0
Total	14.0		5600.0

8.3.3 Run QC qPCR for HGV Master Mix, see section 9.0. If the QC tests pass, then continue.

8.3.4 Take 3 MicroAmp Fast Optical 96-well reaction plates, add 14 µL of HGV Mix into each well.

8.3.5 Cover with optical film, seal it.

8.3.6 Spin at 3,000 rpm for 3 min.

8.3.7 Put the HGV plates in -80°C freezer unit shipping out to EPA lab.

8.4 PCR Master Mix for Enterovirus (EV) Plates: 2 of 96-well plates

8.4.1 One 50 mL centrifuge tube, autoclaved , add 10 mL of RNaseZap and close the cap, vortex for 5 min, then pour off RNaseZap; rinse the tube with 20 mL of DH2O, vortex, repeat three times; and leave the tube in AirClean PCR Workstation for air dry.

8.4.2 Add following EV Master Mix reagents (Table 5) into the treated 50 mL centrifuge tube.

Table 5. Components for qPCR Assay for EV

Ingredient	Volume per RXN (µL)	Final Conc.	Volume per Master (µL)
2X LightCycler 480 Probes Master Mix	10.0	Proprietary	2080.0
ROX reference dye	0.4	0.5 mM	83.2
PCR grade water	1.0		208.0
10 µM EntF	0.6	300 nM	124.8
10 µM EntR	1.8	900 nM	374.4
10 µM EntP	0.2	100 nM	41.6
Total	14.0		2912.0

8.4.3 Run QC qPCR test for EV qPCR Master Mix, see Section 9.0. If the QC tests pass, continue.

8.4.4 Take 2 MicroAmp Fast Optical 96-well reaction plates, add 14 µL of EV Master Mix into each well.

8.4.5 Cover with optical film, seal it.

8.4.6 Spin at 3,000 rpm for 3 min.

8.4.7 Put the EV plates in -80°C freezer unit shipping out to EPA lab.

8.5 PCR Master Mix for Norovirus GIB (GIB) Plates: 2 of 96-well plates

8.5.1 One 50 mL centrifuge tube, autoclaved , add 10 mL of RNaseZap and close the cap, vortex for 5 min, then pour off RNaseZap; rinse the tube with 20 mL of DH2O, vortex, repeat three times; and leave the tube in AirClean PCR Workstation for air dry.

8.5.2 Add following GIB Master Mix reagents (Table 6) into the treated 50 mL centrifuge tube.

Table 6. Components for qPCR Assay for NV-GIB

Ingredient	Volume per RXN (µL)	Final Conc.	Volume per Master Mix (µL)
2X LightCycler 480 Probes Master Mix	10.0	Proprietary	2080.0
ROX reference dye	0.4	0.5 mM	83.2
PCR grade water	0.3		62.4
10 µM NorGIBF	1.0	500 nM	208.0
10 µM NorGIBR	1.8	900 nM	374.4
10 µM NorGIBP	0.5	250 nM	104.0
Total	14.0		2912.0

8.5.3 Run QC qPCR test for GIB qPCR Master Mix, see Section 9.0. If the QC tests pass, continue.

8.5.4 Take 2 MicroAmp Fast Optical 96-well reaction plates, add 14 µL of GIB Master Mix into each well.

8.5.5 Cover with optical film, seal it.

8.5.6 Spin at 3,000 rpm for 3 min.

8.5.7 Put the GIB plates in -80°C freezer unit shipping out to EPA lab.

8.6 PCR Master Mix for Norovirus GII (GII) Plates: 2 of 96-well plates

8.6.1 One 50 mL centrifuge tube, autoclaved , add 10 mL of RNaseZap and close the cap, vortex for 5 min, then pour off RNaseZap; rinse the tube with 20 mL of DH2O, vortex, repeat three times; and leave the tube in AirClean PCR Workstation for air dry.

8.6.2 Add following GII Master Mix reagents (Table 7) into the treated 50 mL centrifuge tube.

Table 7. Components for qPCR Assay for NV-GII

Ingredient	Volume per RXN (µL)	Final Con.	Volume per Master Mix (µL)
2X LightCycler 480 Probes Master Mix	10.0	Proprietary	2080.0
ROX reference dye	0.4	0.5 mM	83.2
PCR grade water	0.3		62.4
10 µM NorGIIF	1.0	500 nM	208.0
10 µM NorGIIR	1.8	900 nM	374.4
10 µM NorGIIP	0.5	250 nM	104.0
Total	14.0		2912.0

8.6.3 Run QC qPCR test for GII qPCR Master Mix, see Section 9.0. If the QC tests pass, continue.

8.6.4 Take 2 MicroAmp Fast Optical 96-well reaction plates, add 14 µL of GII Master Mix into each well.

8.6.5 Cover with optical film, seal it.

8.6.6 Spin at 3,000 rpm for 3 min.

8.6.7 Put the GII plates in -80°C freezer unit shipping out to EPA lab.

9.0 Quality Control Tests for RT Mix-1, RT-Mix-2, HGV, EV, GIB and GII Master Mixes

9.1 The QC tests need to be approved by EPA. The QC tests will run all RT-qPCR assays using Armored RNA standards (Armored EV RNA, Armored GIB RNA and Armored GII RNA) which will be provided by EPA.

9.2 Use 0.1 mL PCR tubes from Life Tech, run RT on ABI Veriti Thermal Cycler.

9.3 Set up QC run for RT following the steps below in the Table 8.

Table 8. QC Test-RT Reactions Setup

Steps	RT Reaction	- Extra HGV RNA	- EV RNA	+ EV RNA	-GIB RNA	+ GIB RNA	-GII RNA	+ GII RNA
Step-1	RT Master Mix-1	16.5 µL	16.5 µL	16.5 µL	16.5 µL	16.5 µL	16.5 µL	16.5 µL
Step-2	DH2O	6.7 µL	6.7 µL		6.7 µL		6.7 µL	
Step-3	Armored Viral RNA			6.7 µL EV		6.7 µL GIB		6.7 µL GII
Step-4	Vortex -10 sec	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Step-5	Spin - 10 sec	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Step-6	PCR Program-Annealing Run *	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Step-7	Spin- 10 sec	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Step-8	RT Master Mix-2	16.8 µL	16.8 µL	16.8 µL	16.8 µL	16.8 µL	16.8 µL	16.8 µL
Step-9	Vortex - 5 sec	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Step-10	Spin - 10 sec	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Step-11	PCR Program RT Run**	Yes	Yes	Yes	Yes	Yes	Yes	Yes

*PCR Program-Annealing Run:	Heat-99°C for 4 min, cool down to 4°C
**PCR Program RT Run:	25°C for 15 min, then 42°C for 60 min, and 99°C for 5 min, cool down to 4°C on hold

9.4 After the RT run, 40 µL of RT product for each reaction is ready for qPCR.

9.5 Use 6 µL of each RT product (6 µL of DH2O for negative controls) for each qPCR test.

9.6 Use 14 µL of each qPCR Master Mix for HGV, EV, GIB and GII.

9.7 Total volume for qPCR is 20 µL.

9.8 qPCR negative controls without RT products are included.

9.9 StepOnePlus Real-time PCR System is used for the qPCR runs.

9.10 Run qPCR for each RT product in triplicates, use Table 9.

9.11 Use 96-well PCR plate; see the Table on next page.

9.12 PCR Program: 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec, and 60°C for 1 min.

Table 9. qPCR Run Setup for QC Tests

	HGV	-EV	+EV	-GIB	+GIB	-GII	+GII
	1	2	3	4	5	6	7
A	HGV-RT	-EV-RT	+EV-RT	-GIB-RT	+GIB-RT	-GII-RT	+GII-RT
B	HGV-RT	-EV-RT	+EV-RT	-GIB-RT	+GIB-RT	-GII-RT	+GII-RT
C	HGV-RT	-EV-RT	+EV-RT	-GIB-RT	+GIB-RT	-GII-RT	+GII-RT
D	ntc	ntc	ntc	ntc	ntc	ntc	ntc
E	ntc	ntc	ntc	ntc	ntc	ntc	ntc
F	ntc	ntc	ntc	ntc	ntc	ntc	ntc

10.0 QC Data Assessment

10.1 **Acceptance Criteria for QC Measures:** all QC data must be maintained and available for easy reference and inspection.

10.1.1 All qPCR runs for RT products with viral RNA should be positive; qPCR runs for all RT products without viral RNA and negative controls (ntc) must be negative.

10.1.2 If QC tests fail, run QC with original reagents. Then, treat all plastic tubes and plates with RNaseZap and rinse with DH20 three times. If QC tests still fail, report to EPA for the failure of QC tests on original reagents. The failure of original reagents in RT-qPCR can be caused by shipping and handling.

10.2 **Corrective Actions:** corrective action policies are addressed in the QA manual.

10.3 **Contingencies for Handling out-of Control or Unacceptable Data:** any quality control requirements not met must have an explanation to their nonconformance.

11.0 Pollution Prevention / Waste Management

11.1 **Pollution Prevention:** EMSL Analytical makes all efforts to reduce the volume and toxicity of the waste generated by the laboratory. An effort to manage procurement of hazardous materials has been implemented in order to avoid over ordering. Hazardous waste is classified for proper disposal.

11.2 **Waste Management:** the waste generated during prep and analysis will be disposed of following safety procedures outlined in the chemical hygiene plan.

12.0 References

12.1 EPA: Statement of Work dated at 7/10/2014, Revised at 1/5/2015, provided by Dr. Shay Fout.

12.2 EPA Method 1615: Measurement of Enterovirus and Norovirus Occurrence in Water by Culture and RT-qPCR, V1.1, revised January 2012.

13.0 Revision History

Revision #	Date	Revision	Initials
0	1/8/2015	Initial version of SOP	C. Li

Authorizing Signatures

Charles Li, Ph.D.		1/8/2015
Author (print)	Author Signature	Date
Reviewer (print)	Reviewer Signature	Date
Corporate Approval (print)	Corporate Approval Signature	Date

SOP Review and Acknowledgement Form

Instructions: Ensure all personnel in laboratory affected by this procedure read and acknowledge. Once completed, the page shall be retained in an Acknowledgments binder along with personnel training.

Laboratory Manager Acknowledgement

My signature below signifies that I have read and understand the entire contents of this document. My signature represents that I agree to fully comply with, implement, and enforce all requirements, procedures, and protocols specified in these procedures set forth in this document and any supporting reference materials or methodologies.

Laboratory Manager Signature _____ Date _____

Laboratory Staff Acknowledgement

My signature below signifies that I have read and understand the entire contents of this document. My signature represents that I agree to fully comply with, implement, and enforce all requirements, procedures, and protocols specified in these procedures set forth in this document and any supporting reference materials or methodologies.

Signature	Date